PATENT
Attorney Docket 2183-6055US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EV348041475US

Date of Deposit with USPS: July 31, 2003

Person making Deposit: Matthew Wooton

APPLICATION FOR LETTERS PATENT

for

ENVIRONMENTALLY REGULATED GENES OF *STREPTOCOCCUS SUIS*

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TITLE OF THE INVENTION

ENVIRONMENTALLY REGULATED GENES OF *STREPTOCOCCUS SUIS*

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of PCT/NL02/00073, filed January 31, 2002, designating the United States of America, corresponding to PCT International Publication WO 02/061070 (published in English on August 8, 2002), the contents of which are incorporated herein in its entirety.

TECHNICAL FIELD

[0002] The invention relates to the field of the diagnosis of and vaccination against *Streptococcal* infections, and to the detection of virulence markers of *Streptococci*.

BACKGROUND

[0003] *Streptococcus species*, of which there are a large variety of that cause infections in domestic animals and man, are often grouped according to Lancefield's groups. Typing according to Lancefield occurs on the basis of serological determinants or antigens that are, among others, present in the capsule of the bacterium and only allows for an approximate determination. Often, bacteria from a different group show cross-reactivity with each other, while other *Streptococci* cannot be assigned a group-determinant at all. Within groups, further differentiation is often possible on the basis of serotyping. These serotypes further contribute to the large antigenic variability of *Streptococci*, a fact that creates an array of difficulties within diagnosis of and vaccination against *Streptococcal* infections.

[0004] Lancefield group A *Streptococcus* (GAS, *Streptococcus pyogenes*) are common with children and cause nasopharyngeal infections and complications thereof. Animals, such as cattle, are susceptible to GAS, wherein mastitis is often found associated with the cattle.

[0005] Lancefield group B *Streptococcus* (GBS) are most often seen with cattle and cause mastitis. However, human infants are susceptible as well, often with fatal consequences. Group B *Streptococci* (GBS) constitute a major cause of bacterial sepsis and meningitis among

human neonates born in the United States and Western Europe and are emerging as significant neonatal pathogens in developing countries.

[0006] Lancefield group C infections, such as those with *S. equi*, *S. zooepidemicus*, *S. dysgalactiae*, and others are mainly seen associated with horses, cattle and pigs, but can also cross the species barrier to humans.

[0007] Lancefield group D (*S. bovis*) infections are found with all mammals and some birds, sometimes resulting in endocarditis or septicemia.

[0008] Lancefield groups E, G, L, P, U and V (*S. porcinus*, *S. canis*, *S. dysgalactiae*) are found with various hosts and cause neonatal infections, nasopharyngeal infections or mastitis.

[0009] Within Lancefield groups R, S, and T (and with ungrouped types), *S. suis* is found and is an important cause of meningitis, septicemia, arthritis and sudden death in young pigs. Incidentally, *S. suis* can also cause meningitis in man.

[0010] Ungrouped *Streptococcus species*, such as *S. mutans*, causes carries with humans. *S. uberis* causes mastitis with cattle, *S. pneumonia* causes major infections in humans, and *Enterococcus faecilalis* and *E. faecium* further contribute to the large group of *Streptococci*. *Streptococcus pneumoniae* (the pneumococcus) is a human pathogen causing invasive diseases, such as pneumonia, bacteremia, and meningitis.

[0011] Little is known about the pathogenesis of the disease caused by *Streptococci*. Various cellular components, such as muramidase-released protein (MRP), extracellular factor (EF) and cell-membrane-associated proteins including fimbriae, hemagglutinins, and hemolysin have been suggested as virulence factors. However, the precise role of these protein components in the pathogenesis of the disease remains unclear. It is known and generally accepted that the polysaccharidic capsule of various *Streptococci* and other gram-positive bacteria play an important role in pathogenesis. The capsule enables these microorganisms to resist phagocytosis and is, therefore, regarded as an important virulence factor or marker.

[0012] In particular, *Streptococcus suis* is an important cause of meningitis, septicemia, arthritis and sudden death in young pigs. It can also cause meningitis in man. Attempts to control the disease are hampered by the lack of sufficient knowledge about the pathogenesis of the disease and the lack of effective vaccines and sensitive diagnostic methods.

[0013] So far, 35 serotypes of *S. suis* are described. Virulence of *S. suis* can differ within and among serotypes. Worldwide, *S. suis* serotype 2 is the most frequently isolated serotype. Within *S. suis* serotype 2, pathogenic, weak-pathogenic and non-pathogenic strains can be found. The pathogenic strains cause severe clinical signs of disease in pigs and large numbers of bacteria can be re-isolated from the central nervous system (CNS) and the joints after experimental infection. The weak-pathogenic strains cause only mild clinical signs of disease and infrequently are bacteria re-isolated from the CNS and the joints after experimental infection. The non-pathogenic strains are completely avirulent in young pigs after experimental infection.

[0014] The 136-kDa muramidase-related protein (MRP) and the 110-kDa extracellular factor (EF) are generally considered as important virulence markers for *S. suis* serotype 2 strains isolated in Europe and the United States. However, differences in virulence between pathogenic, weak-pathogenic and non-pathogenic strains cannot exclusively be explained by differences in their MRP and EF expression patterns. In addition, it is known that the capsule of *Streptococcus suis* serotype 2 is an important virulence factor. However, since pathogenic, weak-pathogenic and non-pathogenic strains seem to be fully encapsulated after growth *in vitro* and *in vivo*, it is not likely that the level of encapsulation of these fully encapsulated strains is associated with their difference in virulence.

SUMMARY OF THE INVENTION

[0015] The invention discloses a method for modulating virulence of a *Streptococcus* comprising modifying a genomic fragment of *Streptococcus*. The genomic fragment comprises at least a functional part of a fragment identifiable by hybridization in *Streptococcus suis* to a nucleic acid or fragment thereof as shown in SEQ ID NOS: 8 through 45 and obtaining a clone including the modified genomic fragment. In one exemplary embodiment, the genomic fragment comprises at least a functional part of a gene, the expression of which can be environmentally regulated in *S. suis* by iron-restricted conditions. In another exemplary embodiment, the genomic fragment comprises at least a functional part of a gene which is expressed in a wild-type *S. suis* infected pig (*in vivo*). In a further exemplary embodiment, selection under iron-restricted conditions is combined with selection *in vivo*. In one embodiment, the gene encodes a

fibronectin/fibrinogen-binding protein. The method disclosed herein is useful for modulating virulence of *Streptococcus suis* and comprises functionally deleting the expression of at least the functional part of the gene by *Streptococcus*.

[0016] The phrase "functionally deleting" as used herein refers to any technique known in the art (such as allowing for a deletion, insertion, mutation or the occurrence of a frame-shift in the open-reading frame of the respective gene) that is instrumental in hampering or inhibiting the expression of a gene-product (be it mRNA and/or protein) of the gene. Thus, the invention discloses a clone of a *Streptococcus* obtained or obtainable by a method according to the invention.

[0017] To get insight in the differences between pathogenic, weak-pathogenic and non-pathogenic strains or clones that are determined by their difference in virulence, the invention describes the identification of environmentally regulated genes of *Streptococcus suis* by iron-restricted conditions and by experimental infection of piglets. Eighteen unique iron-restricted induced (*iri*) genes and 22 unique *in vivo* selected (*ivs*) genes of *S. suis* were found. None of the *ivs* genes was exclusively expressed *in vivo*. Four *iri* genes were substantially identical to four *ivs* genes selected in piglets. Two *ivs* genes were similar to genes for putative virulence factors. One of these *ivs* genes was substantially identical to the *epf* gene of virulent *S. suis* serotype 2 strains and the other *ivs* gene showed homology to a gene encoding a fibronectin-binding protein of *Streptococcus gordonii*.

[0018] In yet another embodiment, the invention discloses a study of the characteristics of fibronectin- and fibrinogen-binding protein of *Streptococcus suis* (FBPS) and its gene as identified herein. The ability to bind fibronectin, either in fluid phase or immobilized onto a surface, is a property of *S. suis* and is one of the mechanisms *S. suis* uses for attachment to and invasion of host cells. Therefore, FBPS is an important virulence factor. The gene encoding FBPS was identified using an *in vivo* selection system in pigs as described herein, thus, showing an important role of the protein *in vivo*. This finding was supported by the observation that isogenic FBPS mutants, herein also disclosed, of *S. suis* are attenuated in pigs. Surprisingly, FBPS bound to fibronectin, as well as to fibrinogen, but did not show the structural characteristics of the fibronectin-binding proteins most commonly described and explains why FBPS has not been found earlier. Most fibronectin-binding proteins described to date are large

cell surface proteins with predicted sizes of 60-100 kDa and have similar structural organizations. The proteins contain an N-terminal signal sequence as well as the cell wall signaling sequence (LPXTGE) (SEQ ID NO: 1). The Fn-binding sites include 30-42 amino acid long motifs, repeated 3-4 times. In particular, the first fibronectin- and fibrinogen-binding protein of *S. suis* is disclosed herein. The gene encoding FBPS was cloned and sequenced and FBPS was purified. Binding of FBPS to human fn and fgn was shown. FBPS was shown to be involved in the colonization of the organs specific for an *S. suis* infection in piglets, but not in the colonization of *S. suis* on the tonsils of piglets.

[0019] Many *Streptococci* and *Staphylococci* have several different fibronectin- and/or fibrinogen-binding proteins, most of which are very large, about 130 kDa. Until now, *S. pyogenes* was the only organism to have a large, as well as a smaller (54 kDa), FnBP. The existence of more than one FnBP explains why in some organisms, isogenic mutants defective in only one of the FnBPs can still bind to fn and/or fgn can be further attenuated *in vivo* in relation to fibronectin binding.

[0020] The role of FBPS in the pathogenesis of *S. suis* was studied in an experimental infection model in piglets. Since we were unable to determine a LD_{50} values for the mutant clones because no lethal dose could be established using normally used numbers of bacteria, it was decided to compare the virulence of the isogenic FBPS clone to the wild-type *S. suis* in a competitive infection assay in piglets. This kind of co-colonization experiment has been successfully applied to determine the virulence of mutants of *Actinobacillus pleuropneumoniae* in piglets. The data showed that the mutant clone was capable of colonizing the tonsil as efficiently as the wild-type. This strongly indicates that FBPS is not involved in the colonization of the tonsil. The data also indicated that FBPS does play a role in the colonization of specific organs, since in the competition assay, joints and the CNS were more efficiently colonized by wild-type than by mutant bacteria.

[0021] In addition, higher numbers of wild-type bacteria were re-isolated from the specific organs compared to the numbers of mutant bacteria, indicating that the mutant clone is attenuated *in vivo*. Although the number of pigs used for this experiment was low, the data indicates that the FBPS mutant is less virulent than the wild-type strain. It was demonstrated that FBPS reacted with a convalescent serum of a pig that survived an *S. suis* infection. Therefore,

FBPS is immunogenic in pigs and this finding demonstrates that FBPS of *S. suis* is expressed under *in vivo* conditions.

[0022] It is also shown that the *fbps* gene was present in all known serotypes of *S. suis* (except for two), as well as in all three phenotypes of serotype 2. This suggests that the *fbps* gene is present among most serotypes. However, the expression of FBPS in all serotypes and phenotypes was not studied. Therefore, it is possible that although all strains, except for serotypes 32 and 34, possess the *fbps* gene, not all strains express FBPS. Based on the facts that FBPS is immunogenic in pigs and that the *fbps* gene is present in all prevailing *S. suis* serotypes, (except for two), FBPS is an attractive candidate for a cross-protective vaccine against essentially all serotypes. In one embodiment, the mutant strain 10ΔFBPS may be used in the vaccine, which mutant is possibly further attenuated by deleting one or more virulence factors as described herein. In another embodiment, this vaccine is based on purified FBPS protein or an antigenic part thereof with a suitable adjuvant.

[0023] To further validate a method for identifying a virulence factor, the role of the fibronectin-/fibrinogen-binding protein (FBPS) in the pathogenesis of *S. suis* serotype 2 was investigated in piglets as described herein. The complete gene encoding FBPS from *S. suis* serotype 2 was cloned in *E. coli* and sequenced. The occurrence of the gene in various serotypes was analyzed by hybridization studies. The FBPS protein was expressed in *E. coli*, purified and binding to human fibronectin and fibrinogen was demonstrated. The induction of antibodies in piglets was studied upon infection. An isogenic mutant unable to produce FBPS was constructed and the virulence of the wild-type and mutant strains was compared in a competitive infection model in young piglets. Organ cultures showed that FBPS was not required for colonization of the tonsils, but that FBPS played a role in the colonization of the specific organs involved in an *S. suis* infection. Therefore, the FBPS mutant was considered as an attenuated mutant which is useful in a vaccine. Alternatively, a vaccine is used that mainly includes the FBPS protein or at least of an antigenic part thereof, such that an FBPS-specific antibody or T-cell response in pigs is developed after vaccination with the FBPS or antigenic part thereof.

[0024] Two additional *ivs* genes showed homology to environmentally regulated genes previously identified by using an *in vivo* expression technology (IVET) selection in other

bacterial species. One of these showed similarity to the *agrA* gene of *Staphylococcus aureus*, a key locus involved in the regulation of numerous virulence proteins.

[0025] Thus, the invention also discloses a method for assaying virulence of a *Streptococcus* comprising assaying a genomic fragment of *Streptococcus,* wherein the genomic fragment comprises at least a functional part of a fragment identifiable by hybridization in *Streptococcus suis* to a nucleic acid or fragment thereof as described herein.

[0026] The invention also discloses a vector comprising a nucleic acid according to the invention and a host cell comprising a nucleic acid or a vector according to the invention. Such a host cell comprises an easily modifiable organism such as *E. coli*. However, other host cells, such as a recombinant *Streptococcus* comprising a vector. or nucleic acid according to the invention are also disclosed herein.

[0027] The invention additionally discloses a vaccine comprising a nucleic acid, a vector or a host cell according to the invention, and use of such a vaccine in the prevention and/or treatment of *Streptococcal* infections.

[0028] Also disclosed is a protein or fragment thereof encoded by a nucleic acid according to the invention, such as a protein encoded by a nucleic acid or fragment thereof disclosed herein or functional, *i.e.*, antigenic fragment thereof. The invention also discloses an antibody directed against a protein or fragment thereof according to the invention and an antigen reactive with such an antibody, for example comprising a protein or fragment. Such a protein or fragment thereof need not be obtained by recombinant means. Synthesis of peptides, according to their amino acid sequence, is as well equally possible. Such antigens and antibodies as described herein can be used in a diagnostic test comprising an antibody according to the invention, or within a vaccine or diagnostic test comprising an antigen according to the invention. Such vaccines and diagnostic tests can be used in the field of the diagnosis of and vaccination against *Streptococcal* infections and for the detection of virulence markers of *Streptococci*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] FIG. 1 is a schematic presentation of the procedure used to clone the *fbps* gene of *S. suis* serotype 2 and the construction of an insertional knock-out mutant in *S. suis* serotype 2.

A 5 kb *Eco*RI fragment was cloned in pGEM7Zf(+), yielding pFBPS7-46. In pFBPS7-47, the 382 bp *SalI-SalI* fragment of pFBPS7-46 was replaced by 1.2 kb spectinomycin-resistance gene, after the vector was made blunt to obtain an insertional knock-out of *fbps*. *Ivs-31*: *in vivo* selected gene 31.

[0030] FIG. 2 shows purity and immunogenicity of FBPS purified under native conditions. SDS-PAGE analysis with SYPRO orange, a non-specific protein-staining (panel A) and Western blot analysis with a monoclonal antibody against the 6 x HIS tag (panel B) of 4:1 of *E. coli* M15 (pQE-3O-pREP4-FBPS) lysate (lanes 1) and 165 ng of purified FBPS (lanes 2). Convalescent serum raised against *S. suis* strain 10 was used to test immunogenicity of FPBS present in 4:1 of *E. coli* M15 (pQE-30-pREP4-FBPS) lysate and 0.5 μg of purified FBPS (Panel C, lanes 1 and 2). Arrowhead, 64 kDa FPBS; Mw, molecular weight marker.

[0031] FIG. 3 depicts the binding studies with purified FBPS. Panels A and B were probed with 5 μg/ml of fn (A) or fgn (B). Lanes 1 contain 500 ng of purified FBPS, lanes 2 contain 500 ng of BSA. Panels C and D, lanes 3 and 4 contain 500 ng of purified FBPS. Lanes 3 were probed with 20 μg/ml of fn (C) or fgn (D), lanes 4 were incubated with conjugate without fn or fgn. Panels E and F were probed with 20 μg/ml of fn (E) or fgn (F). Lanes 5 contain 1.8 μg of purified FBPS digested with enterokinase, lanes 6 contain 500 ng of purified FBPS. The closed arrowhead indicates 64 kDa FBPS; the open arrowhead indicates approximately 55 kDa FBPS without 6 x HIS.

[0032] FIG.4 shows the distribution of *fbps* among various *S. suis* serotypes. 1 μg of chromosomal DNA was spotted onto nitrocellulose membrane and hybridized with a ^{32}P-labelled *fbps* probe. Serotypes were spotted as indicated. S10: *S. suis* serotype 2, MRP^+EF^+; T15: *S. suis* serotype 2 MRP^-EF^-; S17: *S. suis* serotype 2 MRP^+EF^*.

[0033] FIG. 5 illustrates the efficiency of colonization of wild-type and mutant bacteria on various organs of infected pigs. Panel A depicts colonization of the wild-type strain 10 and the mutant strain 10ΔFBPS of the tonsils. A closed diamond symbol is tonsil pig no. 4664; ■ tonsil pig no. 4665; ▲ tonsil pig no 4666; ● tonsil pig no. 4668. Panel B depicts colonization of the specific organs. Open and closed diamond symbols are pus from joints pig no. 4664; ▲ pus from joint pig no. 4666; ● CNS pig no. 4668. Each dot represents the numbers of wild-type or mutant bacteria isolated from one particular organ, from one piglet.

DETAILED DESCRIPTION

[0034] *Streptococcus suis* is an important cause of meningitis, septicemia, arthritis and sudden death in young pigs (Clifton-Hadley, 1983; Vecht *et al.*, 1985). It can also cause meningitis in man (Arends and Zanen, 1988). Attempts to control the disease are still hampered by the lack of sufficient knowledge about the pathogenesis of the disease, the lack of effective vaccines and sensitive diagnostic methods. To meet these shortages, it is necessary to identify the genes that are involved in the pathogenic process. So far, only a limited number of *S. suis* genes are known (Smith *et al.*, 1992; Smith *et al.*, 1993; Serhir *et al.*, 1997; Segers *et al.*, 1998; Smith *et al.*, 1999; and accession nos. AF106927, Z95920 and A57222) and of these, only a few are putatively involved in virulence (Smith *et al.*, 1992; Smith *et al.*, 1993; Jacobs *et al.*, 1994; Gottschalk *et al.*, 1995; Segers *et al.*, 1998; Smith *et al.*, 1999). Previously, putative virulence factors have been identified after growth of the bacteria in standard laboratory media. However, it is known that many important virulence factors are environmentally regulated and are induced at specific stages of the infection process (Mahan *et al.*, 1993).

[0035] Recently, several approaches have been reported that allow the identification of genes that are specifically expressed in the host. Examples are signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET; Mahan *et al.*, 1993; Camilli and Mekalanos, 1995; Hensel *et al.*, 1995; Mahan *et al.*, 1995; Mei *et al.*, 1997; Young and Miller, 1997; Chiang and Mekalanos, 1998; Coulter *et al.*, 1998; Lowe *et al.*, 1998; Polissi *et al.*, 1998; Camacho *et al.*, 1999; Darwin *et al.*, 1999; Edeistein *et al.*, 1999; Fuller *et al.*, 1999; Zhao *et al.*, 1999). In addition, important virulence proteins could also be identified by the selection of genes specifically expressed under conditions mimicking *in vivo* conditions, for example by growth in iron-restricted conditions (Litwin and Calderwood, 1993; Martinez *et al.*, 1990).

[0036] The present invention identifies virulence genes of *S. suis* by selecting environmentally regulated genes by experimental infections of piglets and by the use of iron-restricted conditions *in vitro*. For this purpose, chromosomal DNA fragments of *S. suis* were cloned in a plasmid in front of a promoterless erythromycin-resistance gene. Subsequently, the library was used for the selection of bacteria in which erythromycin resistance was induced under iron-restricted conditions. In addition, erythromycin-resistant bacteria were selected after infection of piglets with the library and treatment of the piglets with erythromycin. Pigs were

used instead of mice for these experiments since it was recently shown that virulence of *S. suis* is different in these two animal species (Vecht *et al.*, 1997). Using this approach, 18 unique iron-restriction-induced (*iri*) genes, as well as 22 unique *in vivo* selected (*ivs*) genes, were identified, several of which are putatively involved in virulence (Smith *et al.*, 1993; Smith *et al.*, 1996).

Methods.

[0037] Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (Oxoid), and plated on Columbia agar (Oxoid) containing 6% (v/v) horse blood. For the selection of genes induced in iron-limited conditions, *S. suis* cells were plated on agar plates containing Todd-Hewitt medium, 5% (w/v) yeast extract and 75 μM deferoxamine mesylate (Sigma). Control plates were supplemented with 38 μM $FeSO_4.7H_2O$ (Sigma). If required, antibiotics were added at the following concentrations: 100 μg spectinomycin ml^{-1} and 1 μg erythromycin ml^{-1}. *E.coli* strains were grown in Luria broth (Miller, 1972) and plated on Luria broth containing 1.5% (w/v) agar. If required, 50 μg ampicillin ml^{-1} or 50 μg spectinomycin ml^{-1} was added.

[0038] Construction of pIVS-E. The IVS selection vector used in this study comprises a spectinomycin-resistance gene, a promoterless erythromycin-resistance gene and the origin of replication of the plasmid pWVO1 (Van der Vossen *et al.*, 1987). To construct this pIVS-E, the spectinomycin-resistance gene was amplified from pKUN19-spc (Konings *et al.*, 1987; Smith *et al.*, 1995). In a PCR reaction, the primers 5′-TGCATGCATGGATCCATCGA TTTTCGTTCG-3′ (SEQ ID NO: 2) and 5′-CGAGCTCGGTACCTGATTACCAATTAGAAT-3′ (SEQ ID NO: 3), which contained *Nsi*I and *Sac*I restriction sites at their respective 5′-ends were used. The obtained PCR product was digested with *Nsi*I and *Sac*I and ligated into pGKV210 (Van der Vossen *et al.*, 1987) that had been digested with *Sac*I (partially) and *Nsi*I. The resulting plasmid was designated pGKV210-spc. pE194 (Horinouchi and Weisblum, 1982) was used as a template for the amplification of a promoterless erythromycin-resistance gene. To do this, the primers 5′-GGGTCGACCCTATAACCAAATTAAAGAGGG-3′ (SEQ ID NO: 4) and 5′-CCCAAGCTTGGGCAGTTTA TGCATCCCTTAAC-3′ (SEQ ID NO: 5) were used in a PCR reaction. These primers contained *Sal*I and *Hind*III restriction sites at their respective 5′-ends. The amplified fragment was digested with *Sal*I and *Hind*III and the fragment was ligated into

pGKV210-spc that had been digested with *Sal*I and *Hind*III. The resulting plasmid was designated pIVS-E. To construct pIVS-PE, the promoter region of the *mrp* gene was inserted into pIVS-E 5′ to the promoterless erythromycin-resistance gene. The promoter region of the *mrp* gene was amplified by PCR from pMRP11 (Smith *et al.*, 1992) using the primers 5′-CCCAAGCTTGGGAATTCATAATGTTTTTTTGAGG-3′ (SEQ ID NO: 6) and 5′-GCGTCGACA TCTACGCATAAAAAATCCCCC-3′ (SEQ ID NO: 7). These primers contained *Eco*RI and *Sal*I sites at their respective 5′-ends. Amplified DNA was digested with *Eco*RI and *Sal*I and the resulting fragment was ligated into *Eco*RI and *Sal*I-digested pIVS-E.

[0039] Construction of genomic S. suis libraries in pIVS-E. *Alu* I partial digests of *S..suis* serotype 2 strain 10 DNA were size fractionated (500-1000 bp) on a 0.8% (w/v) agarose gel. The purified fragments were ligated to *Sma*I and calf intestinal phosphatase digested pIVS-E and the ligation mixtures were transformed to *E. coli* XL2-blue cells. Spectinomycin-resistant colonies were selected. Analysis of the transformants by PCR showed that more then 80% contained an insert. From 15 pools of about 2000-3000 independent *E. coli* transformants, plasmid DNA was isolated. This plasmid DNA was subsequently used for the electrotransformation of *S. suis* strain 10 (Smith *et al.*, 1995). This resulted in approximately 30,000 independent *S. suis* transformants. The transformants were pooled and stored at -80°C.

[0040] DNA techniques. Routine DNA manipulations and PCR reactions were performed as described by Sambrook *et al.* (1989). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems). Samples were prepared by using the ABI/PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Custom-made sequencing primers were purchased from Life Technologies. Sequencing data were assembled and analyzed using the McMollyTetra software package. The BLAST program was used to search for protein sequences similar to the deduced amino acid sequences.

[0041] PCR reaction mixtures (50 µl) contained 10 mM Tris-HCI, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each of the four deoxynucleotide triphosphates, 1 µM of each of the primers and 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems). DNA amplification was carried out in a Perkin Elmer 9600 thermal cycler and the program included an incubation for ten minutes at 95°C and 30 cycles of one minute at 95°C, two minutes at 56°C and two minutes at 72°C.

[0042] Assessment of erythromycin levels in treated piglets. One-week-old specific pathogen-free (SPF) piglets were treated orally with erythromycin stearate (Abbott, 20 or 40 mg body weight kg^{-1}) or intramuscularly with erythromycin (Erythrocin 200; Sanofi Santé, 20 or 40 mg body weight kg^{-1}). Blood samples were collected 3 hours, 6 hours or 24 hours after the administration of the antibiotics to determine erythromycin levels.

[0043] Experimental infections. Gnotobiotic Great Yorkshire and Dutch Landrace crossed piglets were obtained from sows by cesarian section. The surgery was performed in sterile flexible film isolators. The piglets were allotted to groups, each having 4 piglets, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described (Vecht *et al.*, 1989; Vecht *et al.*, 1992). One-week-old piglets were inoculated intravenously with *S. suis* strain 10 (pIVS-E), 10 (pIVS-PE) or 10 (pIVS-RE) as described (Vecht *et al.*, 1989; Vecht *et al.*, 1992, Table 3). Two hours after infection, the pigs were injected intramuscularly with erythromycin for the first time and thereafter received erythromycin twice a day: once intramuscularly (Erythrocin, 40 mg body weight kg^{-1}) and once orally (erythromycin stearate, 40 mg body weight kg^{-1}).

[0044] Piglets were monitored twice a day for clinical signs of disease, such as fever, nervous signs and lameness. Blood samples were collected three times a week from each pig. Leukocyte concentrations were determined using a conducting counter (Contraves A. G., Swizerland). To monitor infection with *S. suis* and to check for absence of contaminants, swabs of the nasopharynx and of feces were collected daily. The swabs were directly plated onto Columbia agar containing 6% (v/v) horse blood. After the piglets were killed, they were examined for gross pathological changes. Tissue specimens were collected from the central nervous system, serosae, joints, lungs, heart and tonsils. The tissues were homogenized in the presence of Todd-Hewitt medium using an Ultra-Turrax tissuemizer (Omni International) and frozen at -80°C in the presence of 15% (v/v) glycerol.

Results.

Promoter selection system.

[0045] The plasmid pIVS-E was constructed to allow introduction of *S. suis* DNA fragments into a number of unique restriction sites in front of a promoterless erythromycin-

resistance gene. The plasmid carries the origin of replication of pWVO1, which functions in *E. coli* and in *S. suis* (Smith *et al.*, 1995). *S. suis* strain 10 cells containing pIVS-E were sensitive to 1 μg erythromycin ml^{-1} on agar plates. In pIVS-PE the promoter of the *mrp* gene of *S. suis* (Smith *et al.*, 1992), which is highly expressed *in vivo* as well as *in vitro*, drives expression of the erythromycin-resistance gene. *S. suis* strain 10 cells containing pIVS-PE were resistant to high concentrations of erythromycin (>256 μg erythromycin ml^{-1}) on agar plates. A *S. suis* DNA library in pIVS-E (pIVS-RE) was constructed and 30,000 individual *S. suis* clones or mutants were obtained. As determined by analysis of 24 randomly selected transformants, more than 80% of these clones or mutants contained an insert (results not shown). Moreover, 2% of the clones were resistant to 1 μg erythromycin ml^{-1} on agar plates indicating the presence of some promoter sequences that were functional *in vitro*.

Selection of promoters induced under iron-restricted conditions.

[0046] Gene sequences that were specifically induced on agar plates under iron-restricted conditions were selected. For this purpose, about 96,000 c.f.u. were plated under iron-limiting conditions on agar plates containing deferoxamine mesylate and erythromycin. The 1500 colonies that grew on these plates were inoculated onto plates containing erythromycin, deferoxamine mesylate and $FeSO_4$. Twenty-four clones showed reduced growth in the presence of $FeSO_4$. The inserts of the 24 selected *iri* clones were amplified by PCR using primers complementary to the 5′ ends of the erythromycin- and spectinomycin-resistance genes and the nucleotide sequences of these fragments were determined. The sequence data showed that the 24 clones contained 18 unique sequences. The 18 sequences were analyzed for similarity to known genes by comparison with the sequences in the GenBank/EMBL and SWISSPROT databases. One sequence, *iri31*, was identical to *cps2A*, a previously identified *S. suis* gene putatively involved in the regulation of capsule expression (Smith *et al.*, 1999). Fourteen *iri* sequences were similar to sequences of known, non-*S. suis*, genes. Three of these sequences (*iri2* (SEQ ID NO: 15), *iri1*, *6* and *22* (SEQ ID NO: 8), and *iri34* (SEQ ID NO:21) were similar to sequences of environmentally regulated genes previously selected by applying the IVET to *V. cholerae* (Camilli and Mekalanos, 1995), *S. aureus* (Lowe *et al.*, 1998) and *P. aeruginosa* (Wang *et al.*, 1996), respectively. One, contained in *iri1*, *6*, and *22* (SEQ ID NO: 8), was similar to the *agrA*

gene of *Staphylococcus aureus*, a key locus involved in the regulation of numerous virulence proteins. Three *iri* sequences had no significant similarity to any sequences in the databases (Table 2).

Conditions for selection of promoter sequences in piglets.

[0047] To determine the antibiotic treatment regime required for a successful selection of *in vivo*-expressed promoter sequences, piglets were treated with different concentrations of erythromycin once a day. The erythromycin was administered either orally or intramuscularly. Levels of erythromycin in sera were determined 3, 6 or 24 hours after treatment over one week. High erythromycin levels were detected three hours and six hours after both treatments (results not shown). However, 24 hours after the treatments, the levels decreased dramatically. Based on these data, we hypothesized that for efficient promoter selection, it was necessary to treat the animals twice a day with erythromycin (40 mg kg^{-1}), once intramuscularly (at 9 a.m.) and once orally (at 4 p.m.).

[0048] To test this hypothesis, pigs were inoculated either with *S. suis* strain 10 (pIVS-PE) or with strain 10 (pIVS-E). In pIVS-PE, the promoter of the *mrp* gene of *S. suis* (Smith *et al.*, 1992), which is highly expressed *in vivo* as well as *in vitro*, drives expression of the erythromycin-resistance gene. The control plasmid, pIVS-E, does not contain a promoter in front of the erythromycin-resistance gene. The strains were inoculated intravenously or intranasally. All pigs infected with strain 10 (pIVS-PE) showed specific *S. suis* symptoms (Table 3) and, except for one, all pigs died in the course of the experiment. Moreover, high numbers of bacteria were isolated from the central nervous system, the serosae and the joints. In contrast, none of the pigs inoculated with strain 10 (pIVS-E) showed specific clinical signs of disease and all survived the infection until the end of the experiment. Moreover, bacteria were not isolated from the central nervous system, the serosae or the joints of these animals. These data demonstrated that *in vivo*-expressed sequences could be selected from pigs using the applied antibiotic treatment regimen.

Selection of gene sequences expressed in vivo in piglets.

[0049] Piglets were inoculated intravenously with different doses (5×10^5 to 5×10^8 c.f.u.) of the *S. suis* library (Table 3) and treated with erythromycin as described herein. Specific signs of disease developed in all animals three to eight days after infection (Table 3). High numbers of bacteria were recovered from tissues (central nervous system, joints, serosae, lung, liver, spleen, heart and kidney) of the individual piglets. Analysis of the recovered bacteria showed that a limited number of different clones were present in each of the bacterial samples isolated from the diseased pigs. For example, 30 randomly selected clones from the joints of one pig all possessed identical DNA inserts as assessed by PCR and DNA sequence analysis (results not shown). In addition, at 80% of the 62 sample sites analyzed, four randomly selected clones were identical. However, from different tissues of a single animal, different clones or mutants could be isolated. On the other hand, identical clones could be isolated from different, as well as from corresponding, tissues of different animals. These findings indicated that a limited number of clones had been selected *in vivo* and were greatly enriched in the affected tissues. The observed selection was not tissue specific. Further, none of the selected clones failed to grow on agar plates that contained 1 μg erythromycin ml^{-1}.

[0050] Two-hundred forty-five clones were analyzed by PCR and partial sequence analysis. Among these, 22 unique *ivs* clones were found. The 22 sequences were analyzed for similarity to sequences of known genes by comparison with the GenBank/EMBL and SWISSPROT databases (Table 4). The sequences of two genes showed similarity to genes encoding putative virulence factors: *ivs21*, *26* and *30* which was identical to the *epf* gene, a previously identified *S. suis* gene, putatively involved in virulence (Smith *et al.*, 1993; Smith *et al.*, 1996); and *ivs31* (SEQ ID NO: 37), which was similar to the fibronectin-binding protein of *S. gordonii*. Moreover, the sequences of two *ivs* genes (*ivs25* (SEQ ID NO: 24) and *ivs6*, *7*, *13* and *14* (SEQ ID NO: 43)) were homologous to two environmentally regulated *ivi* genes, previously identified using IVET selection in other bacterial species (Camilli and Mekalanos, 1995; Lowe *et al.*, 1998). Four *ivs* sequences (*ivs25* (SEQ ID NO: 34); *ivs23* and *24* (SEQ ID NO: 33), *ivs2*, *4* and *28* (SEQ ID NO: 31); and *ivs6*, *7*, *13* and *14* (SEQ ID NO: 43)) were also found when the library was selected using iron-restricted conditions. The remainder of the sequences showed similarity to sequences of known, non-*S. suis* genes, including two genes

showing similarity to mobile elements and five genes showing similarity to genes of unknown function.

[0051] The identification of environmentally regulated genes of *S. suis* serotype 2 by the use of iron-restricted conditions and by experimental infection of piglets is described. Eighteen unique *iri* genes and 22 unique *ivs* genes were found. None of the *ivs* genes was exclusively expressed *in vivo*. Four *iri* genes were identical to four clones selected *in vivo*. The selected gene sequences encode for potential virulence factors, expand our knowledge about the pathogenesis of *S. suis* infections in pigs and are of value in control of the disease either by the development of effective vaccines or by the development of new diagnostic methods. A promoter trap was used to identify environmentally regulated *S. suis* genes expressed under specific conditions, *i.e.*, during iron-restriction or during experimental infection. This system differs from the antibiotic-based IVET system described for *S. typhimurium* (Mahan *et al.*, 1995) in two ways. One is that the *lacZ* reporter gene fusion is omitted in our vector constructions because inclusion of the *lacZ* gene resulted in structural instability of the vector. The other difference is that a plasmid system was used rather than a chromosomal integration system. A plasmid system was used because the low transformation efficiency of *S. suis* (Smith *et al.*, 1995) might prevent the generation of a complete gene library using a chromosomal integration system.

[0052] From the data, it is evident that a number of inducible and environmentally regulated sequences were selected. Four *iri* genes were identical to four *ivs* genes. Because most bacteria require iron for their growth and because there is a limited amount of free iron available within the host (Payne, 1993), it might be expected that the expression of some *ivs* genes is regulated by iron. With the *in vivo* selection system, tissue-specific colonization was not observed: clones isolated from one piglet were also isolated from other piglets from corresponding as well as from different tissues. This might be due to the mechanisms involved in the molecular pathogenesis of *S. suis* infections in pigs. Furthermore, it was striking and different from the observations made with IVET systems that only a limited number of clones could be selected. In addition, we were not able to demonstrate that we selected for gene sequences that are exclusively expressed *in vivo*. This could be explained either by the absence

of promoter sequences exclusively expressed *in vivo* among the 22 identified *ivs* genes, and/or by the inability of this plasmid-based system to identify such sequences due to gene dose effects.

[0053] A number of interesting genes were selected. Two *ivs* genes showed similarity to genes encoding putative virulence factors. *Ivs21*, *26* and *30* were shown to be identical to the *epf* gene of *S. suis* (Smith *et al.*, 1993), which is found in virulent strains of *S. suis* serotypes 1 and 2 (Stockhofe-Zurwieden *et al.*, 1996; Vecht *et al.*, 1991; Vecht *et al.*, 1992). *Ivs31* (SEQ ID NO: 37) showed similarity to the fibronectin/fibrinogen-binding protein of *S. gordonii* (accession no. X65164) and group A *Streptococci* (Courtney *et al.*, 1994). In *Streptococci*, fibronectin/ fibrinogen-binding proteins play an important role in adhesion to host cells and are considered to be important virulence factors. The selection of these two *ivs* genes demonstrated the selectivity of the system and might be indicative for the relevance of the other *ivs* genes in the pathogenesis of *S. suis* infections in pigs. The performance of the system was further demonstrated by the observation that two *ivs* genes, *ivs25* (SEQ ID NO: 34) and *ivs6*, *7*, *13* and *14* (SEQ ID NO: 43) showed similarity to environmentally regulated genes previously identified using an IVET selection system in other bacterial species.

[0054] *Ivs25* (SEQ ID NO: 34) showed significant similarity to the *sapR* gene of *S. mutans* (accession no. P72485) and *Lactobacillus sake* Lb706 (Axelsson and Holck, 1995) as well as to the *agrA* gene of *S. aureus* (Projan and Novick, 1997), both of which encode response regulator proteins of bacterial two-component signal-transduction systems, thus mediating the response to an environmental signal (Projan and Novick, 1997). Use of an IVET selection system for *S. aureus* in mice selected the region preceding the *agrA* gene, suggesting induction of *agrA* expression under *in vivo* conditions (Lowe *et al.*, 1998). Moreover, in *S. aureus,* the *agr* locus was shown to play an important role in altering the expression of a considerable number of virulence factors in response to cell density (Projan and Novick, 1997).

[0055] Clones *ivs6*, *7*, *13* and *14* (SEQ ID NO: 43) showed similarity to a gene, *iviVI*, previously identified by IVET selection in *V. cholerae* (Camilli and Mekalanos, 1995). The function of *iviVI* is unknown. However, the genes showed similarity to members of the ATP-binding cassette family of transporters. The sequenced portion of *ivs6*, *7*, *13* and *14* (SEQ ID NO: 43) included an N-terminal ATP-binding Walker A box motif, which is highly conserved in this transporter family.

[0056] Four *ivs* genes were identical to four *iri* genes. The first gene, *ivs23* and *24* (SEQ ID NO: 33), which is identical to *iri24* (SEQ ID NO: 17), showed similarity to *cpsY* of *S. agalactiae* (Koskiniemi *et al.*, 1998) and to *oxyR* of various organisms (Demple, 1999). *CpsY* of *S. agalactiae* is involved in the regulation of capsule expression and environmental induction of expression of the *cpsY* gene has been suggested by Koskiniemi *et al.* (1998). In *S. suis, ivs23* and *24* (SEQ ID NO: 33).and *iri24* (SEQ ID NO: 17) are not linked to the capsular locus (Smith *et al.*, 1999). The *oxyR* gene is the central regulator of oxidative stress response in *E. coli* (Demple, 1999) and approximately ten genes are under the control of the *OxyR* protein. The second gene, *ivs2, 4* and *28* (SEQ ID NO: 31), which is identical to *iri10* and *20* (SEQ ID NO: 9), showed similarity to the *yoaE* gene of *E. coli* (accession no. P76262), a putative ABC transporter protein. The third and the fourth genes, *ivs25* (SEQ ID NO: 34) and *ivs6, 7, 13* and *14* (SEQ ID NO: 43) were identical to *iri1, 6* and *22* (SEQ ID NO: 8) and *iri2* (SEQ ID NO: 15), respectively. These genes also showed similarity to *ivi* genes selected using IVET in other bacterial species.

[0057] Based on data presented by Niven *et al.* (1999), selection of *iri* genes of S. *suis* is not expected. The authors described that *S. suis* does not require iron for growth. However, in their studies the authors used media reduced from iron by using ethylenediamine di-o-hydroxyphenylacetic acid (EDDA). Therefore, the different conditions used *in vitro* may explain the different results obtained.

[0058] Two of the *S. suis ivs* genes, *ivs1* (SEQ ID NO: 25) and *ivs8* (SEQ ID NO: 44), showed similarity to transposon sequences. Moreover, one *S. suis ivs* gene, *ivs2, 4* and *28* (SEQ ID NO: 31), had a GC% that was considerably higher than the composition of the rest of the selected genes. It is striking that in *S. typhimurium*, several of the *ivi* clones that are required for full virulence have been found to be associated with mobile elements. Their atypical base composition and codon usage has led to the suggestion that they have been acquired from other bacterial species by horizontal transfer (Conner *et al.*, 1998).

[0059] Our screen also identified five *ivs* genes that showed similarity to sequences encoding proteins of unknown function. These genes are not standard housekeeping or metabolic genes.

[0060] Besides the four *ivs/iri* genes, a considerable number of other *iri* genes have been selected in this study by plating the library under iron-restricted conditions. Interestingly, one of the selected *iri* genes, *iri31*, is identical to the *cps2A* gene of *S. suis*. This gene was previously isolated as a part of the capsular locus of *S. suis* serotype 2 (Smith *et al.*, 1999) and was implicated in the regulation of capsular polysaccharide biosynthesis (Kolkman *et al.*, 1997; Smith *et al.*, 1999). Moreover, because the capsule of *S. suis* is expressed in larger size after *in vivo* growth when compared to growth *in vitro* (Quessy *et al.*, 1994), regulated expression of *cps2A* might be expected. Another *iri* gene, *iri7* (SEQ ID NO: 23), showed similarity to the *rpgG* gene of *S. mutans*. This gene was shown to be required for the biosynthesis of rhamnose-glucose polysaccharide (Yamashita *et al.*, 1999). Because rhamnose is part of the polysaccharide capsule in *S. suis* serotype 2 (Elliott and Tai, 1978), a role of the *iri7* (SEQ ID NO: 23) gene in capsule biosynthesis can be proposed. *Iri34* (SEQ ID NO: 21) showed similarity to the *np 16* gene, previously identified using IVET selection in *P. aeruginosa* and suspected to encode threonine dehydratase activity (Wang *et al.*, 1996). Together with the observation that *4 iri* genes could be selected by the *in vivo* approach, these data show that the *iri* genes encode important virulence factors for *S. suis*.

Contribution of Fibronectin-Binding Protein to Pathogenesis of Streptococcus suis serotype 2.

[0061] *Streptococcus suis* causes severe infections, such as meningitis, septicemia, and arthritis, in piglets. The animals often do not survive the infection (6, 28). Occasionally, *S. suis* causes septicemia and meningitis in humans (3). The pathogenesis of an *S. suis* infection is rarely understood. Sows are symptomless carriers of *S. suis* on their tonsils and pass the bacteria on to their piglets. The piglets cannot cope with the bacteria and subsequently develop the specific symptoms of an *S. suis* infection. Until now, 35 capsular serotypes of *S. suis* have been described (26), but serotype 2 strains are most often isolated from diseased piglets. The capsule is an important virulence factor since piglets infected with an acapsular mutant of *S. suis* serotype 2 strains do not develop any clinical symptoms (22). Bacterial proteins have been suggested to play a role in the pathogenesis as well (2, 26). The expression of murimidase-released protein (MRP), extracellular factor (EF) and suilysin was shown to be strongly associated with pathogenic strains of *S. suis* serotype 2 (1, 29, 30). Since isogenic mutants

lacking MRP and EF and isogenic mutants lacking suilysin were still pathogenic in young piglets, these proteins are not absolutely required for virulence (2, 23). Recently, a new virulence factor was identified (21) by using a complementation approach. The function of this virulence factor in the pathogenesis has to be further investigated.

[0062] Many important virulence factors are environmentally regulated and are induced at specific stages of the infection process (15). To identify these genes in *S. suis*, promoters and their downstream sequences that are "on" during experimental *S. suis* infection of piglets (20) were cloned. Twenty-two *in vivo* selected (*ivs*) genes were found. Two of the *ivs* genes were directly linked to virulence since homology was found to genes in the database that encode for known virulence factors. One of these *ivs* genes (*ivs-21*) was identical to the *epf* gene of virulent *S. suis* serotype 2 strains (30). The other (*ivs-31*) (SEQ ID NO: 37) showed homology to genes encoding fibronectin-/fibrinogen-binding proteins of *Streptococcus gordonii* (GenBank accession no. X65164) and *Streptococcus pyogenes* FBP54 (8). A considerable number of fibronectin-binding proteins of various bacterial species have been shown to be important virulence factors (12). In *S. pyogenes*, FBP54 was shown to be expressed in the human host and to preferentially mediate adherence to human buccal epithelial cells (7). It was shown that the FBP54 protein induces protective immunity against *S. pyogenes* challenge in mice (13).

[0063] A fibronectin-/fibrinogen-binding protein of *S. suis* (FBPS) is described herein and the sequence of *fbps* was determined. Binding studies showed that purified FBPS bound fibronectin and fibrinogen. A contribution of FBPS to the pathogenesis of *S. suis* serotype 2 was found.

Materials and Methods.

[0064] Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 5. *S. suis* strains were grown in Todd-Hewitt broth (code CM 189; Oxoid, Ltd.) and plated on Columbia blood base agar plates (code CM331; Oxoid, Ltd., London, United Kingdom), containing 6% (vol/vol) horse blood. *E. coli* strains were grown in Luria Broth (17) and plated on Luria Broth containing 1.5% (wt/vol) agar. If required, antibiotics were added at the following concentrations: 50 μg/ml of spectinomycin (Sigma, St.

Louis, Mo.) for *E. coli* and 100 μg/ml for *S. suis*, 100 μg/ml of ampicillin (Boehringer, Mannheim, Germany) for *E. coli* and 25 μg/ml of kanamycin (Boehringer) for *E. coli.*

[0065] DNA techniques and sequence analysis. Routine DNA manipulations were performed as described by Sambrook *et al.* (19). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems, Warrington, Great Britain). Samples were prepared by use of an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were assembled and analyzed using the Lasergene program (DNASTAR). The BLAST software package was used to search for protein sequences homologous to the deduced amino acid sequences in the GenBank/EMBL databases.

[0066] Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook *et al.* (19). DNA fragments were separated on 0.8% agarose gels and transferred to GeneScreen Plus hybridization transfer membrane (NEN™ Life Science Products, Boston, USA) as described by Sambrook *et al.* (19). DNA probes of the *fbps* and *spc* genes were labeled with (α-^{32}P)dCTP (3,000 Ci/mmol; Amersham Life Science, Buckinghamshire, Great Britain) by use of a random primed DNA labeling kit (Boehringer). The DNA on the blots was pre-hybridized for at least 30 minutes at 65°C and subsequently hybridized for 16 hours at 65°C with the appropriate DNA probes in a buffer containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA and 7% sodium dodecyl sulphate. After hybridization, the membranes were washed twice with a buffer containing 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 5% sodium dodecyl sulphate for 30 minutes at 65°C and twice with a buffer containing 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 1% sodium dodecyl sulphate for 30 minutes at 65°C. The signal was detected on a phosphor-imager (Storm; Molecular Dynamics, Sunnyvale, Calif.).

[0067] Construction of a fbps knock-out mutant. To construct the mutant strain 10ΔFBPS, the pathogenic strain 10 (27, 29) of *S. suis* serotype 2 was electrotransformed (24) with the plasmid pFBPS7-47. In this plasmid, the *fbps* gene was inactivated by the insertion of a spectinomycin-resistance gene. To create pFBPS7-47 (FIG. 1), the 382 bp *SalI*-*SalI* fragment of pFBPS7-46 was replaced by the 1.2 kb *Eco*RV-*Sma*I fragment of pIC-Spc, containing the spectinomycin resistance gene, after the *Sal*I sites of the vector were made blunt (FIG. 1). After electrotransformation of strain 10 with pFBPS7-47, spectinomycin-resistant colonies were selected on Columbia agar plates containing 100 μg/ml of spectinomycin. Southern blotting and

hybridization experiments were used to select for double cross-over integration events (data not shown).

[0068] FBPS expression construct. To construct an FBPS expression plasmid, the QIA*express* Kit (Qiagen GmbH, Hilden, Germany) was used. The primers corresponded to positions 250 to 272 and from 1911 to 1892 of the *fbps* gene. The sequences of these primers were 5'(GCGGATCCGATGACGATGACAAATCTTTTGACGGATTTTTTTTAC)3' (SEQ ID NO: 46) and 5'(CCCAAGCTTGGGCATGAACTAGATTTTCATGG)3' (SEQ ID NO: 47). The primers contained restriction sites for *Bam*HI and *Hind*III, respectively, to amplify the *fbps* gene from pFBPS7-47. The amplified PCR product was digested with *Bam*HI and *Hind*III and the 1.8 kb *fbps* gene was cloned into pQE-30 digested with *Bam*HI and *Hind*III, yielding pQE-30-FBPS. pQE-30-FBPS was transformed to M15 (pREP4).

[0069] Purification of FBPS. M15 (pREP4) (pQE-3O-FBPS) was used to express and purify the FBPS using the QIA*expressionist*™ (Qiagen). In short, M15 (pREP4) (pQE-30-FBPS) cells were grown exponentially; 1 mM IPTG was added and the cells were allowed to grow another four hours at 37°C. Subsequently, cells were harvested and lysed. The cleared supernatants were loaded onto Ni^{2+}-NTA agarose columns. FBPS containing a 6 x HIS tag was bound to the Ni^{2+}-column. The columns were washed and the protein was eluted. Different buffers were used for native and for denaturing purification. FBPS purified under denaturing conditions was renaturated on a Ni^{2+}-NTA column by using a linear 6 M - 1 M urea gradient in 500 mM NaCl, 20% glycerol and 20 mM Tris-HCl (pH7.4), containing protease inhibitors (25 µg/ml of pefabloc, 0.7 µg/ml of pepstatin, 1 µg/ml of aprotinin, 0.5 µg/ml of leupeptin). All procedures were performed according to the manufacturer's recommendations. The 6 x HIS tag was removed from the protein by incubating purified FBPS in 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$ and 0.5 U of light chain enterokinase (New England Biolabs, Beverly, Mass.) for 16 hours at RT.

[0070] Immunization of rabbits with FBPS. Purified and renaturated FBPS was used to immunize two rabbits. To remove urea, the protein was dialyzed against phosphate buffered saline (136 mM NaCl; 2.68 mM KCl; 8.1 mM Na_2HPO_4; 2.79 mM KH_2PO_4 (pH 7.2)) over night at 4°C. Seven days before immunization, blood was collected from the rabbits to determine the natural titers against FBPS. At day one, those rabbits with negative anti-FBPS titers were

immunized intramuscularly with two times 0.5 ml of 100 μg/ml of FBPS in a water-in-oil emulsion (Specol; ID-Lelystad). At day 28, rabbits were immunized for the second time using the same amount of protein and the same route of immunization. Three weeks after the second immunization, the rabbits were sacrificed and blood was collected. The blood was coagulated and serum was collected and used for immuno detection of FBPS.

[0071] Immunodetection of FBPS. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by standard procedures (19). Proteins in the gel were visualized using SYPRO-orange (Molecular Probes, Sunnyvale, Calif.) staining according to the manufacturer's recommendations. Signals were detected on a phosphor imager (Storm; Molecular Dynamics). A known bovine serum albumin concentration range was used as a standard to calculate the amounts of protein present in the gel. The Molecular Dynamics program was used for the calculations.

[0072] Proteins were transferred to a nitrocellulose membrane by standard procedures (19). The membranes were blocked in Blotto: Tris-buffered saline (TBS) (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 4% skimmed milk and 0.05% Tween 20 at room temperature (RT) for one hour. To detect recombinant purified FBPS, membranes were incubated with a monoclonal antibody against the 6 x HIS tag (Clontech, Palo Alto, CA.) in a 1:10,000 dilution in Blotto-TBS (1:1) at RT for one hour, followed by an incubation with alkaline phosphatase-conjugated anti-mouse antibody in a 1:1,000 dilution in Blotto-TBS (1:1) at RT for one hour. Reactivity of purified FBPS was tested by using a convalescent serum of a pig that had survived an *S. suis* infection. Nitrocellulose membranes were incubated with the polyclonal pig serum in a 1:200 dilution in Blotto-TBS (1:1) at RT for one hour, followed by incubation at RT for one hour with alkaline phosphatase-conjugated anti-swine antibody in a 1:2,000 dilution in Blotto-TBS (1:1). As a substrate, Nitro Blue Tetrazolium (Merck, Darmstadt, Germany) bromochloroindolyl phosphate (Sigma) was used. All washing steps were performed in Blotto-TBS (1:1).

[0073] Fibronectin and fibrinogen binding. Binding studies were performed by indirect Western blotting. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described herein. The membranes were blocked in MPBS: PBS containing 4% skimmed milk and 0.05% Tween 20. Subsequently, the membrane was incubated with 5 μg/ml

of human fibronectin (fn) (Sigma) or 5 μg/ml of human fibrinogen (fgn) (Sigma) in PBS containing 5% fetal calf serum, 2% NaCl, and 0.05% Tween 80 at RT for one hour. To detect bound fibronectin and fibrinogen, the membranes were incubated with horse-radish peroxidase-conjugated anti-fibronectin (DAKO) or anti-fibrinogen (DAKO) antibodies in a 1:1,000 dilution in PBS containing 5% fetal calf serum, 2% NaCl, and 0.05% Tween 80 at RT for one hour. The signal was visualized by using ECL^+ (Amersham Pharmacia Biotech, N. J.) according to the manufacturer's recommendations. Signals were detected on a phosphor imager (Storm; Molecular Dynamics). All washing steps were performed in MPBS-PBS (1:1).

[0074] Experimental infections. Germ-free piglets, cross-breeds of Great Yorkshire and Dutch Landrace, were obtained from sows by cesarean section. The surgery was performed in sterile flexible film isolators. Piglets were allotted to groups of four and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described (27, 29). Six-day-old piglets were inoculated intranasally with about 10^7 cfu of *Bordetella bronchiseptica* 92932 to predispose the piglets to infection with *S. suis*. Two days later, the piglets were inoculated intranasally with 10^6 cfu of *S. suis* strain 10 plus 10^6 cfu of *S. suis* strain 10ΔFBPS. To determine differences in virulence between wild-type and mutant strains, LD_{50} values should be determined. To do this, large numbers of piglets are required. For ethical reasons, this is not acceptable. To circumvent this problem, co-colonization studies were performed.

[0075] To monitor for the presence of *S. suis* and *B. bronchiseptica* and to check for absence of contaminants, swabs taken from the nasopharynx and the feces were cultured three times a week. The swabs were plated directly onto Columbia agar containing 6% horse blood or grown for 48 hours in Todd-Hewitt broth and subsequently plated onto Columbia agar containing 6% horse blood. Pigs were monitored twice a day for clinical signs and symptoms, such as fever, nervous signs, and lameness. Blood samples from each pig were collected three times a week. Leukocytes were counted with a cell counter. The piglets were killed when specific signs of an *S. suis* infection were observed, such as arthritis or meningitis, or when the pigs became mortally ill. The other piglets were killed two weeks after inoculation with *S. suis* and examined the same way as the piglets that were killed based on their clinical symptoms. All piglets were examined for pathological changes.

[0076] Tissue specimens from heart, lung, liver, kidney, spleen, and tonsil, and from the organs specifically involved in an *S. suis* infection (central nervous system (CNS), serosae, and joints) were sliced with a scalpel or a tissuenizer. Tissue slices from each organ or site were resuspended in 2 - 25 ml of Todd-Hewitt containing 15% glycerol depending on the size of the tissue slice. The suspension was centrifuged at 3,000 rpm for five minutes. The supernatant was collected and serial dilutions were plated on Columbia agar containing 6% horse blood, as well as on Columbia agar plates containing 6% horse blood and 100 μg/ml of spectinomycin to quantitate the number of wild-type and mutant bacteria present. The number of mutant strain 10ΔFBPS cells was determined by counting the number of CFU on the appropriate serial dilution on the selective plates; the number of wild-type strain 10 cells was determined by counting the number of CFU on the appropriate serial dilution on the Columbia Agar blood plates of which the number of CFU counted on the selective plates was subtracted. When wild-type and mutant bacteria were found in tissues, the ratio of wild-type and mutant strain was determined again by toothpicking about 100 individual colonies onto both Columbia Agar plates and onto Columbia Agar plates containing 100 μg/ml spectinomycin.

[0077] All animal experiments were approved by the ethical committee of the Institute for Animal Science and Health in accordance with the Dutch law on animal experiments.

[0078] Nucleotide sequence accession number. The nucleotide sequence data of *fbps* have been submitted to GenBank, in which the sequence is listed under accession no. AF438158.

Results.

[0079] Cloning of the *S. suis* fbps gene. One of the *in vivo* selected genes (*ivs-31*) (SEQ ID NO: 37) (20) showed homology to the 5′ part of genes encoding for FlpA and FBP54, fibronectin-binding proteins (FnBP) of *Streptococcus gordonii* (GenBank accession no. X65164) and *Streptococcus pyogenes* (8), respectively. To clone the entire *fbps* gene of *S. suis*, *ivs31* (SEQ ID NO: 37) was used as a probe to identify a chromosomal DNA fragment of *S. suis* serotype 2 containing flanking *fbps* sequences. A 5 kb *Eco*RI fragment was identified and cloned in pGEM7Zf(+) yielding pFBPS7-46 (FIG. 1). Sequence analysis revealed that this fragment contained the entire *fbps* gene of *S. suis* serotype 2.

[0080] An open reading frame of 1659 bp coding for a polypeptide of 553 amino acids was found. The putative ATG start codon is preceded by a sequence similar to ribosome binding sites of gram-positive bacteria. Further upstream, two putative promoter sequences could be identified. Upstream of these promoter sequences of *fbps*, a direct repeat was found that could serve as a transcription terminator of the gene located 5' of *fbps*. Downstream of *fbps*, a gene that showed homology to an alpha-acetolactate decarboxylase was found. This gene is transcribed in the opposite direction of *fbps*. The deduced amino acid sequence was aligned with that of several previously identified FnBPs from other bacteria. As expected, FBPS was substantially homologous to FlpA of *S. gordonii* (76%) and also showed homology to FnBP's of other organisms, like *Streptococcus pneumoniae* (73%), *S. pyogenes* (69%), *Lactococcus lactis* (59%), and *Bacillus subtilis* (41%). Compared to the sequence of FBP54, FBPS has a longer N-terminus with 76 additional amino acids. This longer N-terminus was also seen in other organisms like S. *gordonii*, *S. pneumoniae* and *B. subtilis*. In FBP54, the primary fibronectin-/fibrinogen-binding domain was localized to its N-terminal part, to the first 89 amino acids (8). Over this region, the homology of FBPS to FBP54 is very high (80%), suggesting that FBPS can bind both fibronectin and fibrinogen.

[0081] Binding of FBPS to fibronectin and fibrinogen. To confirm the binding of FBPS of *S. suis* to fibronectin (fn) and fibrinogen (fgn), FBPS was purified under native conditions. A protein expression construct, which expresses FBPS with a 6 x HIS tag fused to the N-terminus, was used for purification. Four hundred μg of FBPS was purified from 50 ml of exponential-phase *E. coli* cells after induction with IPTG. The purity of this FPBS was determined with SDS-PAGE and Western blotting (FIG. 2). The induced *E. coli* lysate contained a broad range of proteins, among which the 64 kDa protein FBPS was present (panel A, lane 1). After purification, highly purified FBPS with 6 x HIS tag was obtained (panel A, lane 2). When both samples were incubated with a monoclonal antibody against the 6 x HIS tag, FBPS was the only protein that was detected (panel B).

[0082] To determine whether FBPS binds fn and fgn, a Western blot containing purified FBPS was incubated with soluble human fn and human fgn (FIG. 3, panels A and B). Specific binding of fn and fgn to FBPS was detected. No binding of fn and fgn to BSA, a negative control protein, was observed. To exclude possible background signals due to

immunoglobulin-binding of FBPS, the same experiment was performed without addition of fibronectin or fibrinogen. No binding was found (FIG. 3, panels C and D) indicating that the binding was specific for fibronectin and fibrinogen. To control whether the binding of fn and fgn to FBPS was not mediated by the 6 x HIS tag, the tag was removed by an enterokinase treatment. FIG. 3, panels E and F, show that FBPS without the 6 x HIS tag still efficiently bound to fn and fgn. Therefore, it appears that FBPS can specifically bind to fn and fgn.

[0083] Immunogenicity of FBPS. Since it was shown that FBP54 induced a protective immune response in mice against a lethal dose of *S. pyogenes* (13), it was determined whether purified FBPS was recognized by convalescent serum of a pig that survived an *S. suis* infection. As shown in FIG. 2 panel C, the FBPS reacted with this anti-serum. When the same experiment was performed with non-immune serum of an SPF piglet, no band of the size of FBPS was detected (data not shown). These findings indicate that FBPS is expressed *in vivo* and that the protein is indeed immunogenic in young pigs.

[0084] Distribution of the fbps gene among the 35 *S. suis* serotypes. Since we were interested in a cross-protective vaccine candidate, the presence of the *fbps* gene among the various *S. suis* serotypes was analyzed. *Ivs-31* (SEQ ID NO: 37), the clone containing the promoter and the 5'-part of the *fbps* gene, was radiolabeled and chromosomal DNA of the reference strains of the 35 different *S. suis* serotypes was hybridized with this probe. The three different phenotypes of *S. suis* serotype 2, a pathogenic, a non-pathogenic and a weak pathogenic strain, were included in this study. The *fbps* gene was present in all *S. suis* serotypes and phenotypes except for serotypes 32 and 34 (FIG. 4).

[0085] Role of FBPS in pathogenesis. To test the role of FBPS in the pathogenesis of *S. suis*, an isogenic knock-out mutant of FBPS was constructed in strain 10, strain 10ΔFBPS. Since upstream of *fbps* a direct repeat was found that could serve as a transcription terminator and downstream of *fbps* a gene showing homology to an alpha-acetolactate decarboxylase was found that is transcribed in the opposite direction, polar effects to genes upstream or downstream of *fbps* are not expected. To verify that the mutant strain 10ΔFBPS did not produce FBPS, protoplasts of strain 10 and strain 10ΔFBPS were subjected to SDS-PAGE and Western blotting. FBPS was detected using a polyclonal antiserum raised against purified FBPS. It was shown that strain 10ΔFBPS expressed no FBPS, while strain 10 did express FBPS (data not shown).

Subsequently, the virulence of this mutant strain was tested in an experimental infection in piglets.

[0086] The mutant strain 10ΔFBPS was used in a competition challenge experiment with the wild-type strain to determine the relative attenuation of the mutant strain. Using *in vitro* conditions, the growth rates of the wild-type and mutant strain in Todd-Hewitt medium were found to be essentially identical (data not shown). Wild-type and mutant strain were inoculated at an actual ratio of 0.65 (1.63×10^6 cfu of wild-type bacteria ml^{-1} and 3.09×10^6 cfu of mutant bacteria ml^{-1}). During the experiment, piglets that developed specific *S. suis* symptoms (meningitis, arthritis, or mortal illness) were killed. Piglets that did not develop these symptoms were killed at the end of the experiment. From all piglets, the ratio of wild-type and mutant strain in various organs was determined.

[0087] As shown in FIG. 5, panel A, similar numbers of wild-type and mutant bacteria were re-isolated from tonsils. The ratio was similar to the input ratio (ratio varied from 0.33 - 0.85, average 0.61). This indicates that the efficiency of colonization of wild-type and mutant strain on tonsils was essentially identical. Apparently, FBPS is not strictly required for colonization of the tonsils of the piglets. Three out of four piglets developed clinical signs specific for an *S. suis* infection. Two piglets (4664 and 4666) showed clinical signs of arthritis and one piglet (4668) showed clear central nervous signs. The fourth piglet did not develop any clinical signs. These observations coincided with pathomorphological abnormalities of the specific organs of an *S. suis* infection in post-mortem sections.

[0088] As shown in FIG. 5, panel B, exclusively wild-type bacteria were re-isolated from the joints of piglet 4664 and from the CNS of piglet 4668. The numbers of CFU of wild-type bacteria that were re-isolated from these specific organs were very high, while no mutant bacteria were found. From the joints of pig 4666, low numbers of both wild-type and mutant bacteria were re-isolated in a ratio of 0.84 (1.0×10^2 CFU of wild-type bacteria and 5.2×10^2 CFU of mutant bacteria), a ratio essentially identical to the input ratio (FIG. 5, panel B). Southern blot experiments using the *fbps* and the *spc* genes as probes, confirmed that the mutant bacteria isolated from the joint of pig 4666 were indeed identical to the input mutant bacteria. Taken together, these data indicate, that the FBPS mutant is capable of reaching and colonizing

the specific *S. suis* organs (at least the joints), but that the mutant is far less efficiently recovered from organs than the wild type.

Table 1. Bacterial strains and plasmids.

Strain/plasmid	relevant characteristics*	source/reference
Strain		
E. coli		
XL2 blue		Stratagene
S. suis		
10	virulent serotype 2 strain	(Vecht *et al.*, 1992)
Plasmid		
pKUN19-spc	replication functions pUC, Amp^R, Spc^R	(Konings *et al.*, 1987, Smith *et al.*, 1995)
pGKV210	replication functions pWVO1, Cm^R, Em^R	(Van der Vossen *et al.*, 1987)
pE194	Em^R	(Horinouchi & Weisblum, 1987)
pMR11	pKUN19 containing *S. suis mrp* gene	(Smith *et al.*, 1992)
pIVS-E	replication functions pWVO1, Spc^R, promoterless *emR* gene of pE194	this work
pIVS-PE	pIVS-E containing promoter of *mrp* preceding the promoterless *emR* gene	this work
pIVS-RE	pIVS-E containing random *S. suis* sequences preceding the promoterless *emR* gene	this work

* Spc^R: spectinomycin resistant
Amp^R: ampicillin resistant
Em^R: erythromycin resistant
Cm^R: chloramphenicol resistant

Table 2. Iron-restriction induced *S. suis* genes.

Clone	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
			Regulatory functions		
iri 1, 6, 22 (SEQ ID NO: 8)	800	34	*S. mutans* SapR (U75483)	response regulator protein	44
			S. aureus AgrA (X52543)	response regulator protein	51
			S. aureus Ivi2		
iri 24 (SEQ ID NO: 17)	850	38	*S. agalactiae* CpsY (CAB36982)	regulation capsule expression	46
			E. coli OxyR (P11721)	oxidative stress regulator	51
iri 23 (SEQ ID NO: 16)	1000	38	*B. subtilis* YvyD (P28368)	sigma-54 modulator homologue	44
			Metabolic functions		
iri 7 (SEQ ID NO: 23)	800	39	*S. mutans* RgpG (Q9XDW8)	rhamnose-glucose biosynthesis	76

Clone	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
iri 11 (SEQ ID NO: 10)	700	34	*L. lactis* NrdD (Q9ZAX6)	anaerobic ribonucleotide reductase	51
iri 14 (SEQ ID NO: 12)	500	38	*S. pneumoniae* SulB (Q54614)	dihydrofolate synthetase	41
iri 16 (SEQ ID NO: 13)	850	48	*B. subtilis* TrmU (O35020)	RNA methyltransferase	62
iri 32 (SEQ ID NO: 20)	300	41	*C. histolyticum* RuvB (O9ZNJ5)	hypoxanthine-guanine phosphoribosyl transferase	55
iri 34 (SEQ ID NO: 21)	1000	44	*L. lactis* IlvA (U92974)	probable threonine dehydratase	56
			P. aeruginosa Pn16		
			Transporter functions		
iri 2 (SEQ ID NO: 15)	750	36	*B. subtilis* YloD (O34328)	putative guanylate kinase	50
			S. gordonii ComYA (U81957)	putative ABC transporter	37
			Vibrio cholerae IviVI (Q56605)	putative ABC transporter	47

Clone	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
iri 10, 20 (SEQ ID NO: 9)	1350	51	*E. coli* YoaE (P76262)	putative transport protein	94
				Unknown	
iri 13, 15, 27 (SEQ ID NO: 11)	800	34	*M. tuberculosis* MTCY336_33	unknown	38
			hypothetical protein (O06593)		
iri 29 (SEQ ID NO: 18)	850	36	*S. aureus* Yp15 (P13977) hypothetical protein	unknown	39
iri 18 (SEQ ID NO: 14)	800	39	*S. crista* hypothetical protein (AAF61316)	unknown	82
iri 3 (SEQ ID NO: 19)	700	36	no homology found		
iri 4 (SEQ ID NO: 22)	700	36	no homology found		

Clone	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
iri 8, 26 (SEQ ID NO: 24)	900	35	no homology found		

Table 3. Virulence of *S. suis* 10 (pIVET-E), 10 (pIVET-PE) and 10 (pIVET-RE) in gnotobiotic piglets.

Strains/ library	No. of piglets	Dose (route of infection)	Mortality * (%)	Morbidity ¥ (%)	Clinical index of the group ¦		Fever index à	Leukocyte index £	No. of pigs from which *S. suis* was isolated		
					Specific ‖	Non-specific ¤			CNS	Serosae	Joints
10 (pIVS-E)	4	10^6 (i.n.)	0	0	0	6	9	75	0	0	0
10 (pIVS-E)	4	10^6 (i.v.)	0	0	6	12	31	0	0	0	0
10 (pIVS-PE)	4	10^6 (i.n.)	100	100	30	40	35	100	3	0	2
10 (pIVS-PE)	4	10^6 (i.v.)	75	100	50	42	43	50	3	3	4
10 (pIVS-RE)	4	5×10^5 (i.v.)	100	100	56	75	44	83	2	2	4
10 (pIVS-RE)	4	5×10^6 (i.v.)	100	100	43	73	43	60	3	0	4
10 (pIVS-RE)	4	5×10^7 (i.v.)	100	100	60	74	48	75	4	1	4
10 (pIVS-RE)	4	5×10^8 (i.v.)	100	100	49	70	37	50	3	3	4

* Percentage of pigs that died due to infection or had to be killed for animal welfare reasons
¥ Percentage of pigs with specific symptoms
¦ Percentage of observations which matched the described criteria
‖ Ataxia, lameness of at least one joint and/or stiffness
¤ Inappetance and/or depression
à Percentage of observations for the experimental group of a body temperature of > 40°C
£ Percentage of blood samples for the group in which the concentration of granulocytes was > 10^{10}/liter

Table 4. *S. suis* genes selected in pigs.

Clone	Sites of isolation	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
				Putative virulence factors		
ivs 31 (SEQ ID NO: 37)	CNS	200	47	*S. gordonii* FlpA (X65164)	fibronectin/fibrinogen binding	70
				Regulatory functions		
ivs 25 (SEQ ID NO: 34)	joint	800	34	*S. mutans* SapR (P72485)	response regulator protein	49
				S. aureus AgrA (X52543)	response regulator protein	51
				S. suis Iri 1, 6, 22		100
ivs 23, 24 (SEQ ID NO: 33)	other	850	38	*S. agalactiae* CpsY (CAB36982)	regulation capsule expression	46
				E. coli OxyR (P11721)	oxidative stress regulator	51
				S. suis Iri 24		100
ivs 16 (SEQ ID NO: 28)	CNS	800	43	*S. epidermidis* AltR (U71377)	putative transcriptional regulator	26
ivs 20 (SEQ ID NO: 32)	lung	800	41	*L. lactis* AldR (O34133)	putative regulator AldR	64

Clone	Sites of isolation	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
				Metabolic functions		
ivs 33 (SEQ ID NO: 39)	CNS	570	36	*E. coli* ThrC (P00934)	threonine synthase	41
ivs 5, 10, 12, 22 (SEQ ID NO: 42)	CNS, joint	900	36	*S. gordonii* Tdk (P47848)	thymidine kinase	87
ivs 18 (SEQ ID NO: 29)	lung	730	32	*S. mutans* NADH oxidase (JC4541)	NADH oxidase	80
				Transporter functions		
ivs 2, 4, 28 (SEQ ID NO: 31)	CNS, joint	1350	51	*E. coli* YoaE (P76262)	putative transport protein	94
				S. suis Iri 10, 20		100
ivs 3 (SEQ ID NO: 36)	joint	1000	42	*S. mutans* OrfU (AF267498)	putative ABC transporter (permease)	33
ivs 6, 7, 13, 14 (SEQ ID NO: 43)	CNS, joint	1350	36	*B. subtilis* Ylo D (O34328)	putative guanylate kinase	50

Clone	Sites of isolation	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
				S. gordonii ComYa (U81957)	putative ABC transporter	37
				V. cholera IviVI (Q56605)	putative ABC transporter	47
				S. suis Iri 2		100
				Transposases		
ivs 8 (SEQ ID NO: 44)	CNS	600	41	*S. pneumoniae* transposase (Z86112)	transposase	70
ivs 1 (SEQ ID NO: 25)	joint	1600	39	*C. perfringens* (X71844)	putative transposase	56
				Miscellaneous		
ivs 32, 35 (SEQ ID NO: 38)	CNS	500	38	*S. typhimurium* FliF (P15928)	flagellar M-protein precursor	36
ivs 9, 17 (SEQ ID NO: 45)	joint, CNS	800	36	*B. subtilis* ComE ORF2 (P32393)	competence development	37
ivs 11 (SEQ ID NO: 26)	serosea	800	44	*P. syringae* TabA (P31851)	diaminopimelate decarboxylase/ tabtoxin	53

Clone	Sites of isolation	Insert (bp)	GC%	Data base homology (accession no.)	Function of homolog	% Identity
					Unknown	
ivs 15 (SEQ ID NO: 27)	CNS	750	42	*B. subtilis* conserved hypothetical protein YdiB (D88802)	unknown	43
ivs 29 (SEQ ID NO: 35)	joint	800	38	*S. salivarius* hypothetical protein (AF130465)	unknown	79
ivs 34 (SEQ ID NO: 40)	CNS	600	43	*B. subtilis* conserved hypothetical protein YrrK (O34634)	unknown	61
ivs 36 (SEQ ID NO: 41)	joint	830	42	*B. subtilis* hypothetical protein YqeG (P54452)	unknown	35
ivs 19 (SEQ ID NO: 30)	lung	950	34	*S. cristatus* hypothetical protein (U96166)	unknown	86

Table 5. Bacterial strains and plasmids.

Strain/plasmid	Relevant Characteristics[a]	Source/reference
Strains		
E. coli		
XL2-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1*	Stratagene
	lac (F' *proAB lacI*^{q}*Z*)M15 TN*10* (Tet^R) *amy* Cm^R)	
M15	Nal^S Str^S Rif^S Thi^- Lac^- Ara^+ Gal^+ Mtl^- F^- $RecA^+$ Uvr^+ Lon^+	Qiagen
S. suis		
10	Virulent serotype 2 strain	Vecht *et al.* (29)
10ΔFBPS	Isogenic *fbps* mutant of strain 10	This work
Plasmids		
pGEM7Zf(+)	Replication functions pUC, Amp^R	Promega Corp.
pKUN19	Replication functions pUC, Amp^R	Konings *et al.* (14)
pIC19R	Replication functions pUC, Amp^R	Marsh *et al.* (16)
pDL282	Replication functions of pBR322 and pVT736-1, Amp^R, Spc^R	Sreenivasan *et al.* (25)
pIC-spc	pIC19R containing Spc^R gene of pDL282	Lab collection
pQE-30	Replication functions pBR322, Amp^R, expression vector, 6x HIS tag	Qiagen

Strain/plasmid	Relevant Characteristics[a]	Source/reference
pQE-30-FBPS	pQE-30 containing the 1.8 kb *fbps* gene	This work
pREP4	Replication functions pACYC, Kan^R, *lac*I gene	Qiagen
pE194	Em^R	Horinouchi and Weisblum (11)
pIVS-E	Replication functions of pWVO1, Spc^R, promoterless *erm* gene of pE194	Smith *et al.* (20)
pIVS-31	pIVS-E containing 200 bp showing homology to *Streptococcus gordonii flpa*	Smith *et al.* (20)
pFBPS7-46	pGEM7Zf(+) containing *Eco*RI-*Eco*RI fragment of *fbps*	This work
pFBPS7-47	pFBPS7-46 in which 382 bp *Sal*I-*Sal*I fragment is replaced by 1.2 kb Spc^R from pIC-spc	This work

[a] Tet^R tetracycline resistant
Cm^R chloramphenicol resistant
Amp^R ampicillin resistant
Spc^R spectinomycin resistant
Kan^R kanamycin resistant
FBPS fibronectin binding protein *S. suis*

Table 6. Numbers of re-isolated wild-type (strain 10) and mutant (strain 10ΔFBPS) bacteria from organs of infected piglets (mean actual inoculation ratio 65% mutant strain).

Organ	Pig number											
	4664			4665			4666			4667		
	w.t.[a]	mut.[b]	perc.[c] mut.	w.t.[a]	mut.[b]	perc.[c] mut.	w.t.[a]	mut.[b]	perc.[c] mut.	w.t.[a]	mut.[b]	perc.[c] mut.
Tonsil	1.77^{e}5	3.29^{e}5	65	4.35^{e}5	2.42^{e}6	85	5.34^{e}4	8.73^{e}4	61	7.94^{e}5	3.96^{e}5	33
pus joint 1	6.75^{e}4	< 10	0				1.02^{e}2	5.2^{e}2	84			
pus joint 2	5.15^{e}4	< 10	0									
CNS										1.88^{e}5	< 10	0

CNS Central Nervous System

[a] number of wild-type bacteria found (cfu/ml)

[b] number of mutant bacteria found (cfu/ml)

[c] percentage of mutant bacteria calculated as follows: b/(a+b) * 100%

Only relevant organs are depicted.

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